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DIAGNOSTICS OF THE STAGES OF POST-EMBRYONIC DEVELOPMENT IN *BURSAPHELENCHUS ULMOPHILUS* (NEMATODA: APHELENCHOIDIDAE)

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The research was aimed to study diagnostic characters distinguishing individual development stages of *Bursaphelenchus ulmophilus* (*Hofmanni* group), which propagative generation worms were multiplied in sterile laboratory cultures of the fungus *Botrytis cinerea*. J2 hatched after the first molt in the egg shell. Postembryonic stages of individual development include 4 stages (J2—J4 and adults) separated by molts. Juvenile stages differ in structure and size of the genital primordium. The genital primordium of J2 consists of 2 large germinal cells and two apical somatic cells; the presence of 2 germinal cells indicates the didelphic female genital tube of the *Bursaphelenchus* ancestor. During the following development, both germinal cells shift into the anterior genital tube, because *Bursaphelenchus* spp. females possess an unpaired genital system with a single anterior branch and anterior ovary. A sex in juveniles may be identified from the J3 stage. In J4, sections of the forming genital system are recognizable. A key to developmental stages of *B. ulmophilus* is given. The body length growth exceeds pharynx growth. The juvenile body increases during molts and within each stage. Comparing to the earlier development, the growth accelerates between stages J3 and J4; in the same development phase, the most active cellular differentiation and elongation of the genital primordium take place. The *B. ulmophilus* dauers taken from the *Scolytus* spp. beetle under-elytra spaces are identical to the third juvenile stage of the propagative generation, whereas in *B. mucronatus* dauers (the species of *Xylophilus* group) are the fourth juvenile stage (J4D). Differences between species are also visible in the absence of the mucro on the tail tip in adults and juveniles of *B. ulmophilus*, whereas a mucro is present in *B. mucronatus* in juveniles of J3 and J4 stages and in females.

Key words: *Bursaphelenchus ulmophilus*, individual development, postembryogenesis, diagnostics, saproxylic nematodes; *Ulmus*, *Scolytus*, Saint Petersburg.

ДИАГНОСТИКА СТАДИЙ ПОСТЭМБРИОНАЛЬНОГО РАЗВИТИЯ ВЯЗОВОЙ НЕМАТОДЫ *BURSAPHELENCHUS ULMOPHILUS* (NEMATODA: APHELENCHOIDIDAE)

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Целью исследования было выявление диагностических признаков, по которым можно отличить стадии индивидуального развития *Bursaphelenchus ulmophilus* (*Hofmanni* group), пропагативное поколение которых было размножено в стерильной лабораторной культуре гриба *Botrytis cinerea.* Первая линька происходит внутри яйцевой оболочки, из яйца выходит личинка второй стадии (J2). В постэмбриональном развитии имеются 4 стадии (J2—J4 и половозрелые самцы и самки), разделенные друг от друга линьками. Стадии личинок отличаются между собой по структуре и размеру полового зачатка. Половой зачаток личинки второй стадии (J2) состоит из двух крупных центральных герминальных клеток и двух мелких апикальных соматических ядер на полюсах зачатка. Наличие двух герминальных клеток в зачатке, каждая из которых может продуцировать яичник, указывает на парную половую систему у самок предка рода. В последующем развитии обе герминальные клетки перемещаются в переднюю ветвь полового зачатка, так как женская половая система бурсафеленхов непарная, она состоит из одной передней трубки. Пол личинки возможно идентифицировать, начиная с третьей личиночной стадии. В половых зачатках у личинок четвертой стадии различимы функциональные отделы. Дан ключ для идентификации стадий развития и их пола для *B. ulmophilus.* Рост тела происходит быстрее, чем рост глотки. Личинки увеличиваются в размерах в ходе линек, но также происходит рост личинки внутри каждой стадии. Наибольший скачок роста наблюдается между третьей и четвертой стадиями. В этой же фазе развития резко увеличивается половой зачаток и происходит его клеточная дифференцировка. Дауеры, личинки инвазионного поколения *B. ulmophilus* после извлечения из нематангиев под элитрами, идентичны по строению полового зачатка личинке третьей стадии пропагативного поколения. Это отличает вязовую нематоду от сосновой нематоды *B. mucronatus* (из группы *Xylophilus*), у которых дауеры в жуке переносчике — это личинки четвертого возраста. Различия между видами также в отсутствии мукро на кончике хвоста *B. ulmophilus* на всех стадиях развития; у *B. mucronatus* мукро имеется у стадий J3 и J4 и у самки.

Ключевые слова: *Bursaphelenchus ulmophilus*, индивидуальное развитие, постэмбриогенез, диагностика, ксилобионтные нематоды, *Ulmus*, *Scolytus*, Санкт-Петербург.

The nematode species *Bursaphelenchus ulmophilus* Ryss, Polyanina, Popovichev et Subbotin, 2015 (*Hofmanni* group) was originally described from the dying elm *Ulmus glabra* Huds. found in the park of the Forestry Technological University (Saint Petersburg), and now it was detected in more than 20 localities across the city (Ryss et al., 2015; Polyanina et al., 2016; Ryss, Subbotin, 2017). All records revealed associations of *B. ulmophilus* with symptoms of Dutch Elm Disease (DED) of *Ulmus laevis* Pall. and *U. glabra*, with bark beetle vectors *Scolytus multistriatus* (Marsham, 1802) and *S. scolytus* (Fabricius, 1775). Transmissive dauer juveniles of *B. ulmophilus* were found in senior larvae, pupae, and female imagoes of the beetle species. From these dauer juveniles, adult ne-

matodes of *B. ulmophilus* were grown in cultures of the spore-less strain of the fungus *Botrytis cinerea* on the 2 % potato dextrose agar (PDA) (Ryss, 2015; Ryss et al., 2015). Earlier, the pathogenic fungus *Ophiostoma novo-ulmi* Brasier, 1991, the causative agent of DED, was detected for the association, with the same beetle vectors (Kalko, 2008, 2009; Fedorova, 2010). It was concluded that *B. ulmophilus* is an associate of DED pathogens with a role of an acceleration of spread of the *O. novo-ulmi* spores along the elm trunk in the phloem layer (Ryss et al., 2015). To study host-parasite and vector-parasite relations of *B. ulmophilus*, the identification of the life cycle stages is necessary. The goal of this research is to identify the juvenile stages of the species. Special attention is given to the genital primordium structures, because according to previous studies, these structures are most useful in identification of stage and sex of juveniles (Hirschmann, 1962, 1971; Hirschmann, Triantaphyllou, 1968; Ryss, 1981).

MATERIAL AND METHODS

The nematodes *Bursaphelenchus ulmophilus* were collected by the junior author in the Moskovsky Victory Park («Moskovskiy Park Pobedy»), a park in the south of Saint Petersburg, on September 9, 2016. GIS: 59°52′06.4′′ N 30°19′45.1′′ E. Host: *Ulmus laevis* with DED symptoms (red-brownish ring in a phloem layer of tree trunk and branches) and the bark beetle *S. multistriatus* imagoes and 2nd stage larvae in bark tunnels in the inner bark layer and softwood. Nematodes were multiplied in the sterile laboratory cultures on the lawn of spore-less strain of the fungus *Botrytis cinerea*, inoculated in the 2 % potato dextrose agar media in Petri dish. Over one week after 100 nematode specimen inoculation, nematodes occupied all the Petri dish space (6 cm in diameter), devastating the fungus lawn, showing active oviposition and population growth. Nematode suspension was washed out with 1 ml of distilled water and fixed in hot TAF as described by Ryss (2017). Over 2 days after fixation, nematodes were stained with acetic orcein. To stain nematodes, a modification of the staining technique (Ryss, 1988; Ryss, Chernetskaya, 2009) was used. A 1.5 ml Eppendorf tube with nematodes in TAF was placed in upright position for 10 min to settle nematodes down, and the upper layer of fixative was removed with a syringe up to the 2 fold level of the nematode suspension height (usually 100 microliters). Then 200 microliters of 70 % acetic acid were added into the tube. After shaking of a tube, two drops of saturated acetic orcein solution in 65 % acetic acid were added, which is used usually for chromosome staining. The mixture was kept overnight for staining procedure and then a drop of staining suspension was placed into a glycerin in a cavity slide. Stained nematodes were picked out with needle under a stereomicroscope and placed in a minute drop of glycerin on a microscopic slide. The drop was covered with 24 × × 24 mm coverslip. The drop was spread under coverslip to press nematodes to obtain flattened worm bodies for detailed study of nematode genital primordia structures. After drop spreading, coverslip was sealed with nail polish along borders.

Measurements and values used in morphometry of the individual development stages. Measures: L: body length; BW: body width; pharynx length till the end of the pharyngeal gland lobe, tail length; tail width at anus (ABW), GP:

genital primordium length; GW: genital primordium width; spicule length along arc; hyaline part of tail in male juveniles (future bursal flap of male). Ratios: a: body length divided to body width; b': body length divided to pharynx length till the end of pharyngeal glands (because pharyngo-intestinal valve is hardly recognizable in juveniles and thus b-value cannot be calculated); c: body length divided to tail length; c': tail length divided to tail width; V or (V): distance from anterior body end to vulva, or to the center of the vulval primordium, divided to the body length, in %; GP/GW: genital primordium length divided to its width; GP/L: genital primordium length divided to body length, in %.

Because the bodies were flattened, the re-calculation of the body and tail diameter was used: the flattened width was considered as a half of the circle (πR or pi R), thus the diameter (2R) was calculated as the flattened body width (BW)*(2/pi) = 0.64*BW.

RESULTS

Orcein-stained nematodes and eggs were studied. The first molt took place in the egg shell; thus during hatching the second stage juvenile (J2) broke the egg-shell and came out (fig. 1).

Other stages may be classified as J2, J3, J4, and adult males and females, separated by three molts. Molts were detected by cuticle separation from the body at body extremities, the head and tail tips (fig. 2). In juveniles J2, J3, and J4 the sexes of juveniles may be recognized whereas in J2 the sex was unknown. The stage and sex of the juvenile may be determined in the genital primordium structure and its position in the body. The body size and the ratio between the body length and the pharynx are useful to identify the stage. Genital primordia in every stage and sex are described and illustrated below.

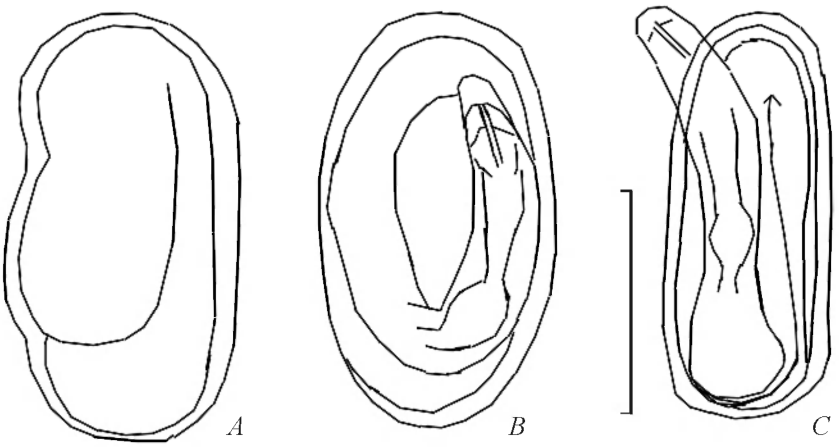

Fig. 1. Egg development.
A — gastrula, *B* — first molt in egg, *C* — hatching. Scale — 25 μm.

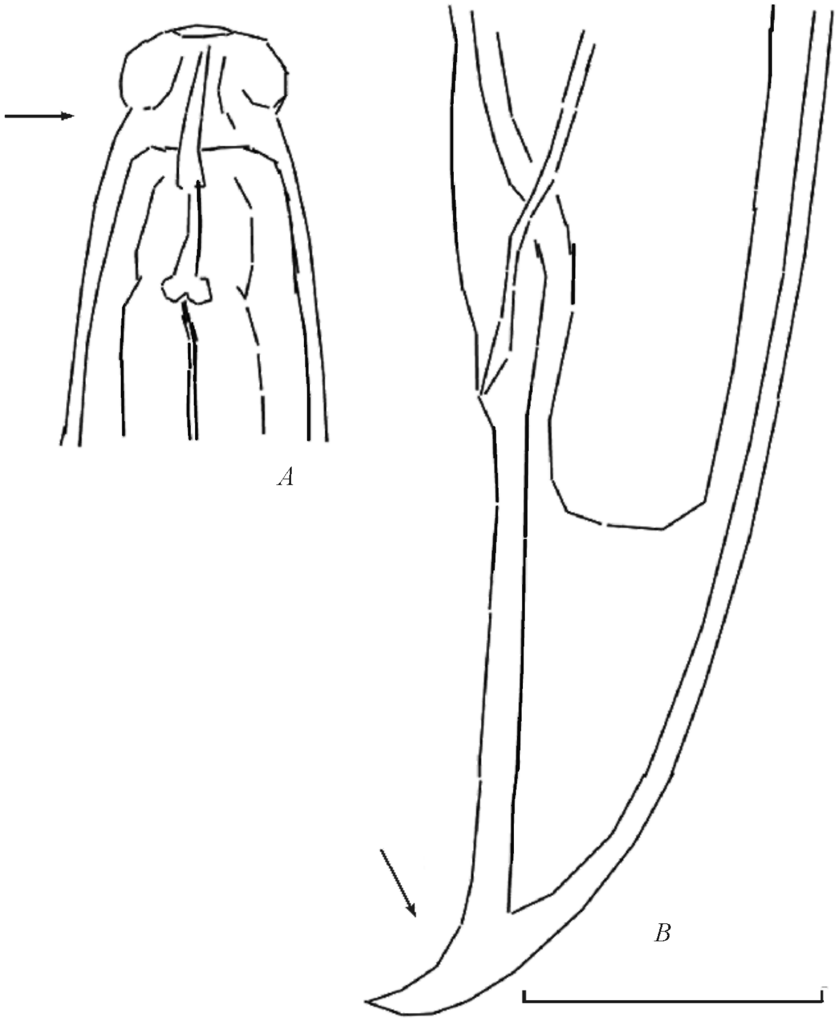

Fig. 2. Molting male juvenile, J4 to adult male.
A — head; *B* — tail tip, with shed cuticle. Arrows: molting cuticle separated from body wall. Scale — 10 µm.

Second stage juveniles (J2)

Fig. 3, *A*, *B*; 5, 6. Measurements are given in the table.

The genital primordium consists of four cells: two large germinal cells in the central part and two small somatic nuclei at extremities (fig. 3, *B*). Primordium is in the middle of the intestine part of the body (fig. 5).

Differences from other stages. J2 differs from senior stages in the smallest number of cells in primordium as well as in the body size less than 200 µm, *vs* body length of 300 µm or longer in juveniles of senior stages.

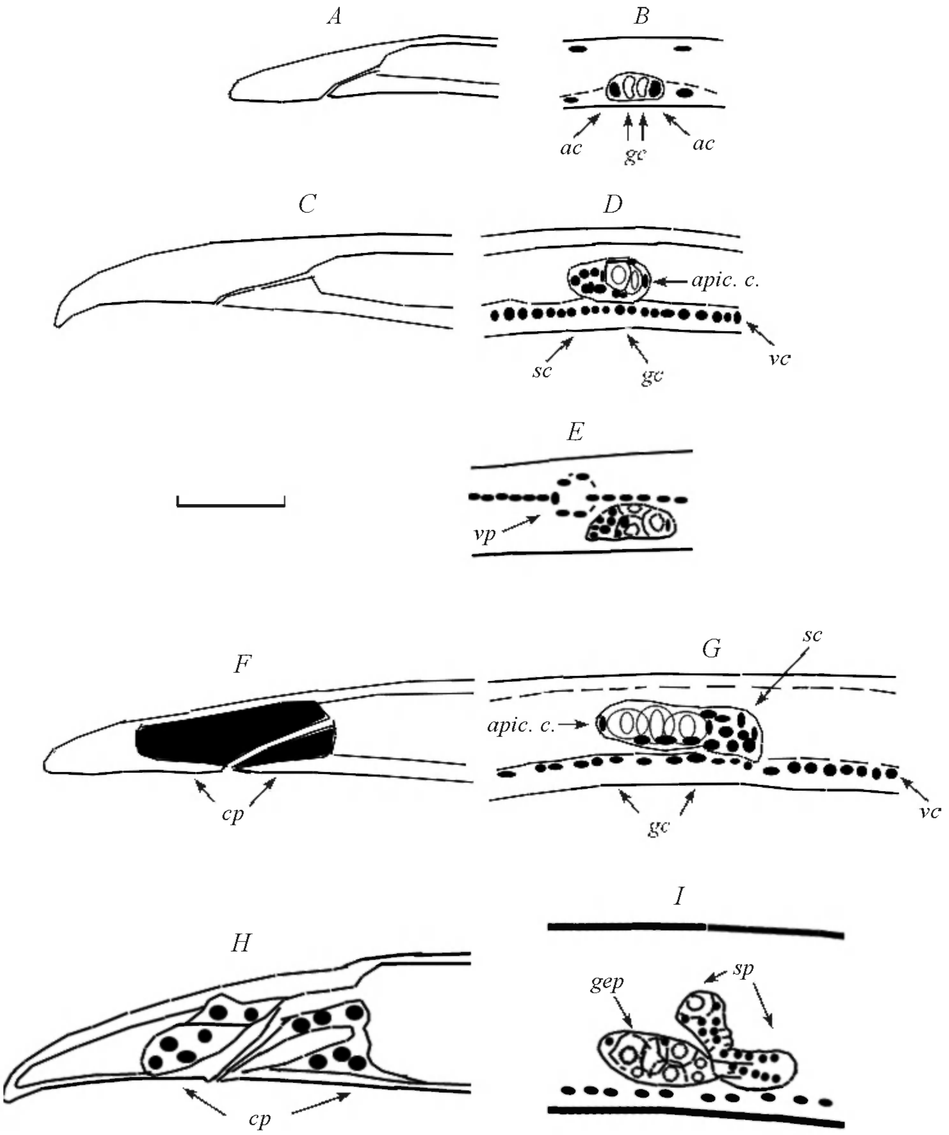

Fig. 3. Juvenile tails (*A, C, F, H*) and genital primordia (*B, D, E, G, I*).

A, B — second stage juvenile; *C, D, E* — third stage juvenile, female (*C, D* — lateral view; *E* — ventral view); *F, G* — third stage male juvenile; *H, I* — molting male juvenile from third stage to fourth stage (*I* — «loop stage» of the male primordium with its somatic part reverse movement). *ac* — apical cells, *cp* — cloaca primordium, *gc* — germinal cells, *gep* — germinal part of primordium, *sc* — somatic cells, *sp* — somatic part of genital primordium, *vc* — ventral cord of hypodermal cells nuclei, *vp* — vulva primordium. Scale — 10 μm.

Third stage juveniles

Third stage male juveniles. Fig. 3, *F, G*; 5. Measurements are given in the Table.

In the genital primordium, 3—5 germinal cells in the posterior part and 12 somatic nuclei in anterior part (fig. 3, *G*) are present. In addition, a single

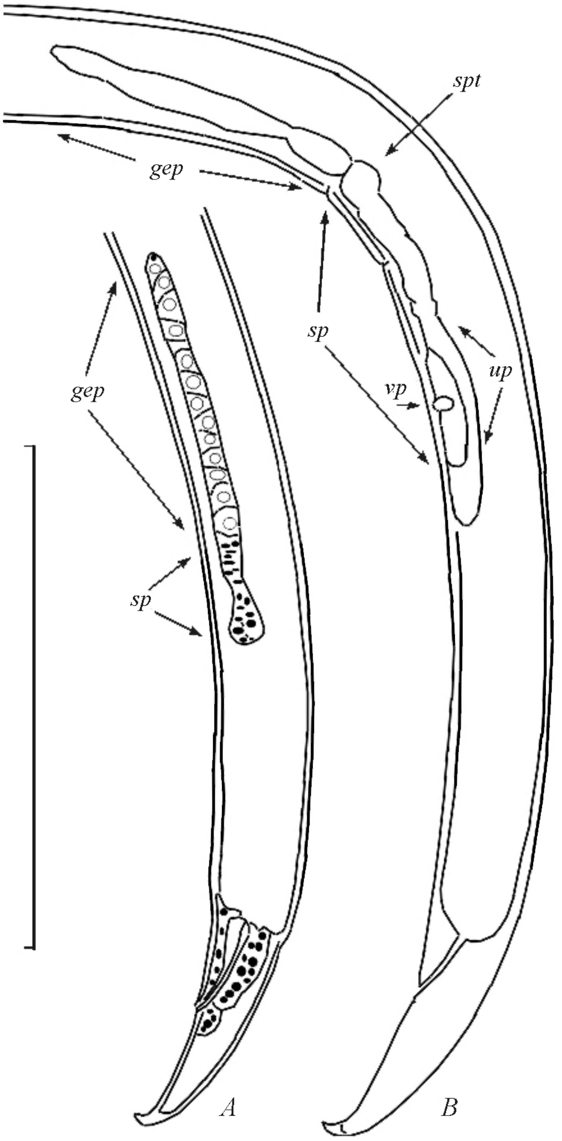

Fig. 4. Genital primordium and tails of the fourth stage juveniles.
A — fourth stage male juvenile, *B* — fourth stage female juvenile. *gep* — germinal part of primordium, *sp* — somatic part, *up* — uterus primordium, *vp* — vulva primordium. Scale — 100 μm.

somatic apical nucleus at every extremity of the primordium is found. The primordium is situated in the middle of the intestine part of the body. Cloaca primordium is present as a dense agglomeration of somatic nuclei around the rectum.

Third stage female juvenile. Fig. 3, *C*, *D*, *E*; 6. Measurements are given in the Table.

In the genital primordium, 2 large germinal cells in the anterior part and 12 somatic nuclei in the posterior primordium part are present together with a single somatic apical nucleus on every extremity of the primordium (fig. 3, *D*, *E*).

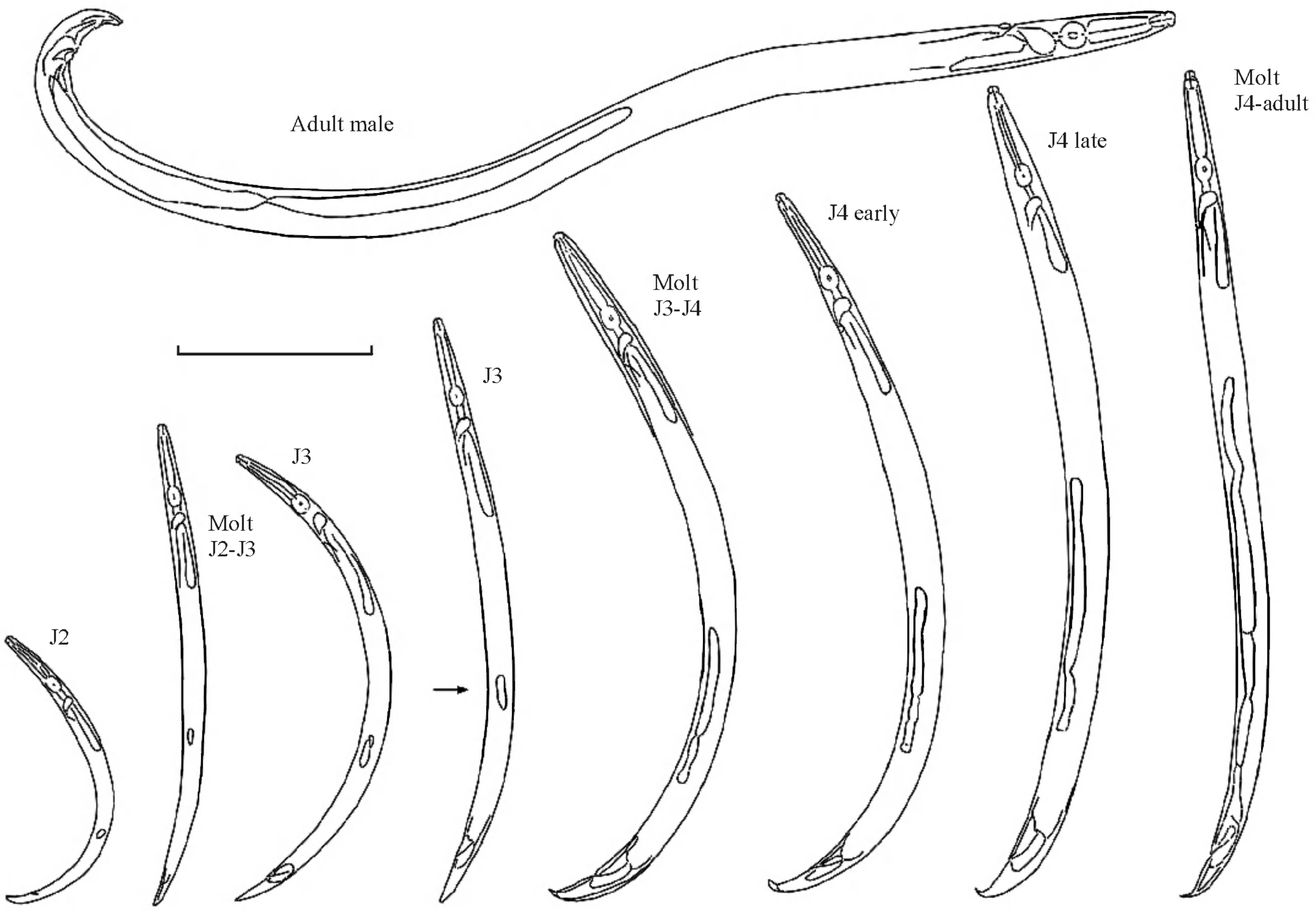

Fig. 5. Position of genital primordia in bodies of male juveniles and adults.
Arrow: genital primordium position. Scale — 100 μm.

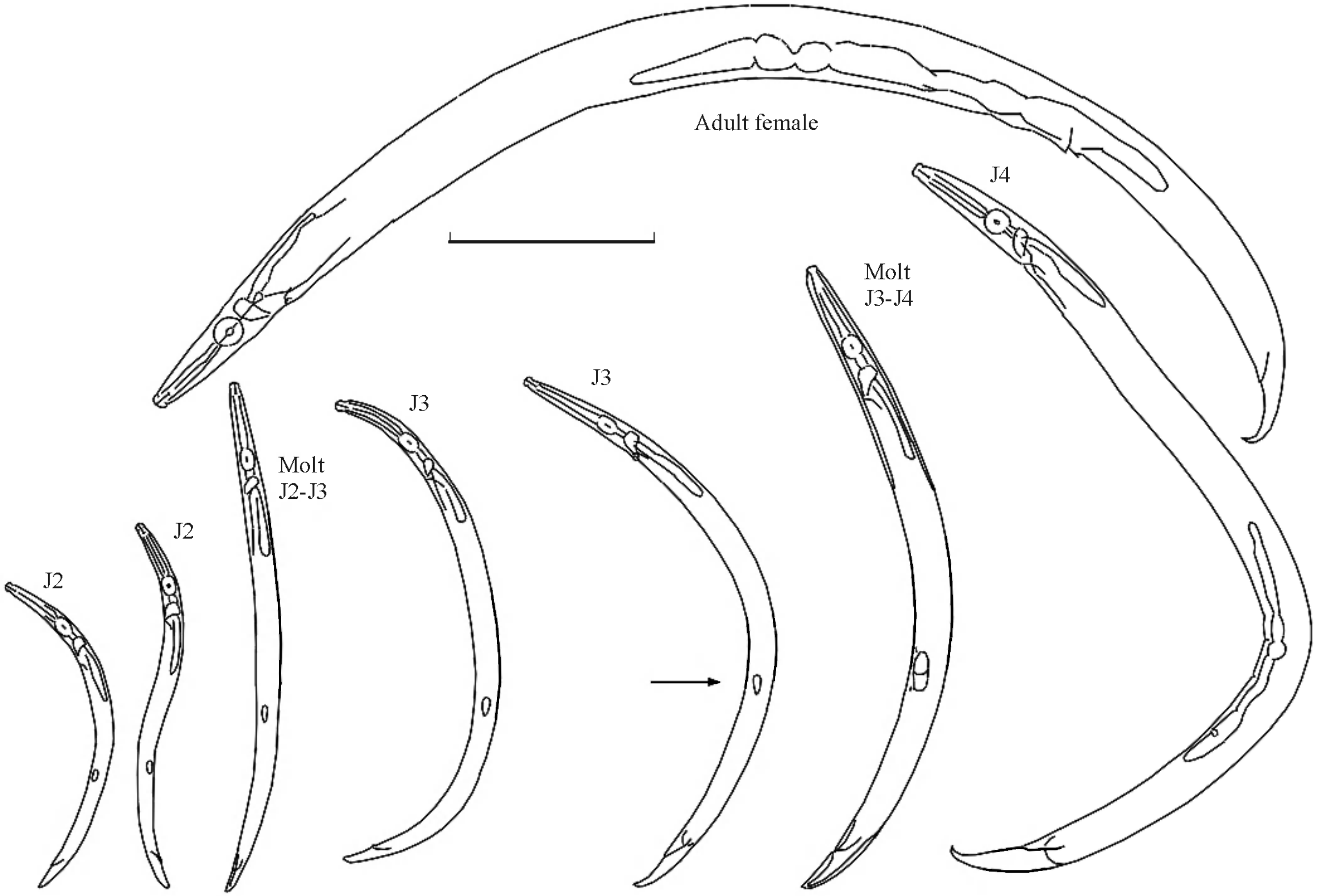

Fig. 6. Position of genital primordia in bodies of female juveniles and adults.
Arrow: genital primordium position. Scale — 100 μm.

Measurements (in μm) and ratios of the development stages of *Bursaphelenchus ulmophilus*

Stage/Character	J2	J3 male	J3 female	J4 male	J4 female	Male	Female
n	20	20	20	20	20	15	15
L	188 ± 8 (179—201)	332 ± 26 (291—362)	347 ± 37 (285—385)	425 ± 11 (413—440)	485 ± 36 (450—520)	679 ± 11 (666—685)	674 ± 90 (538—802)
Body width	6 ± 1 (5—8)	10 ± 1 (9—11)	10 ± 2 (8—13)	15 ± 2 (13—17)	16.4 ± 0.3 (16—17)	15 ± 1 (14—16)	19 ± 3 (14—23)
Pharynx	68 ± 4 (63—74)	97 ± 5 (90—104)	90 ± 6 (81—97)	117 ± 5 (112—124)	110 ± 8 (102—118)	117 ± 8 (108—122)	111 ± 15 (86—130)
Tail	15 ± 2 (14—19)	24 ± 3 (19—27)	24 ± 3 (18—27)	31 ± 2 (29—34)	28 ± 2 (26—30)	30.3 ± 0.4 (29—31)	38 ± 4 (31—43)
Anal body width	4 ± 1 (4—5)	6 ± 1 (5—7)	6 ± 1 (4—7)	7 ± 1 (7—8)	9.0 ± 0.3 (8—10)	10 ± 1 (9—10)	7 ± 1 (6—9)
Genital primordium length (GPL)	5 ± 1 (4—8)	15 ± 2 (11—18)	12 ± 3 (8—18)	105 ± 27 (86—142)	138 ± 1 (136—140)	371 ± 65 (333—445)	252 ± 53 (175—306)
Genital primordium width (GPW)	3 ± 1 (2—4)	4 ± 1 (3—5)	4 ± 1 (3—5)	6 ± 1 (5—7)	8.5 ± 0.5 (8—9)	12 ± 2 (11—14)	16 ± 2 (14—19)
Spicule length along arc	—	—	—	—	—	13 ± 1 (12—14)	—
a	32 ± 5 (25—37)	33.2 ± 0.9 (32—34)	35 ± 4 (28—39)	30 ± 3 (26—32)	30 ± 3 (27—32)	44 ± 3 (42—48)	36 ± 4 (31—42)
b’	2.8 ± 0.2 (2.5—3.1)	3.4 ± 0.3 (3.1—3.9)	3.8 ± 0.3 (3.5—4.5)	3.7 ± 0.2 (3.3—3.9)	4.4—4.5	5.8 ± 0.3 (5.6—6.2)	6.1 ± 0.6 (5.3—7.0)
c	12 ± 1 (10—14)	14 ± 1 (13—16)	15 ± 1 (12—16)	14 ± 1 (12—15)	17 ± 1 (16—18)	22 ± 1 (21—23)	18 ± 2 (15—20)
c’	3.6 ± 0.2 (3.4—4.1)	3.9 ± 0.2 (3.5—4.5)	4.2 ± 0.3 (3.7—4.6)	4.2 ± 0.4 (3.6—4.6)	3.1 ± 0.3 (2.8—3.4)	3.1 ± 0.2 (3.0—3.3)	5.2 ± 0.5 (4.7—6.2)
V % or (V)	66 ± 1 (65—68)	—	62 ± 2 (58—66)	—	73 ± 2 (71—75)	—	73 ± 1 (71—75)
GPL/GPW	1.6 ± 0.2 (2.0—2.4)	4.1 ± 1.0 (2.8—5.7)	2.6 ± 0.6 (2.0—3.9)	17.4 ± 2.5 (14.3—20.3)	16.3 ± 1.0 (15.3—17.3)	30.8 ± 0.4 (30.5—31.3)	16.6 ± 4.5 (11.0—21.8)
GPL/L, %	3 ± 1 (2—4)	4.6 ± 0.9 (3—6)	3.0 ± 0.7 (3—5)	25 ± 6 (20—32)	29 ± 2 (27—31)	55 ± 11 (49—67)	39 ± 11 (26—56)

Note. All values are given as mean ± STD (minimum—maximum).

A circle of six somatic nuclei interrupt the ventral hypodermal cord chain of somatic nuclei (fig. 3, *E*). It is a primordium of vulva.

Differences of the third stage juveniles. Differences of male juvenile of the third stage from female juvenile are manifested in the presence of cloaca primordium and the position of germinal cells in the posterior part of the primordium. In female juveniles, germinal cells are located in the anterior part of the primordium. Third stage juveniles differ from J2 in the number of cells of the primordium (more than 10 *vs* 4 in J2). From J4 juveniles, the third stage juveniles duffer in the length of primordium occupying 6 % of the body length or less *vs* 20 % or more in J4 and adults.

Fourth stage juveniles

Fourth stage male juveniles. fig. 4, *A*; 5. Measurements are given in the Table.

Cloaca primordium is massive, with numerous somatic nuclei around rectum and a transparent cavity anterior to rectum with rudimentary spicules. The genital primordium is distinctly divided into an anterior germinal part of 30—60 large cells and a somatic part of two rows of somatic nuclei with 15—16 nuclei in every row. The somatic part is not divided into sections. A spherical empty cavity is found in the posterior end of the primordium (fig. 4, *A*). Genital primordium occupies 20—32 % of the body length (fig. 5). Tail tip with narrowly conical 6 (4—9) μm hyaline zone curved ventrally, corresponding to bursal flap of adult male.

Fourth stage female juvenile. fig. 4, *B*; 6. Measurements are given in the Table.

Genital primordium is divided into the anterior germinal part, consisting of 20—30 large cells and the posterior somatic part consisting of more than 60 somatic nuclei. The somatic part is divided into sections among which the uterus primordium (in all J4 juveniles) and the spherical spermatheca primordium (only in senior J4 juveniles) are distinct (fig. 4, *B*). The uterus primordium consists of many rows of somatic cells. On the ventral side of body wall in the middle of uterus primordium, the lens-like invagination surrounded by massive structure attached to the ventral body wall, is distinct. It is the vulva primordium. Genital primordium occupies 27—31 % of the body length (fig. 6).

Differences of the fourth stage juveniles. Differences between male juveniles of the fourth stage from female juveniles are manifested in the presence of cloaca primordium and in the absence of sections of the somatic part of the genital primordium *vs* distinct sections in primordium of female juveniles with the massive uterus and spermathecal section. In female juveniles, the lens-like transparent vulva primordium at the level of uterus primordium part is present. In J4 male juveniles, the vulva primordium is absent. The fourth stage juveniles differ from J3 in the larger genital primordium (20 % of body length or more *vs* 6 % or less in J3) and in numerous cells of the primordium: more than 50 in J4 *vs* 20 or less in J3. From adult nematodes, J4 differ in the absence of copulative organs *vs* spicules or vulva are present in adult nematodes.

Adults

Measurements are given in the Table.

Orcein staining did not reveal any new morphological features in addition to detailed description published earlier (Ryss et al., 2015).

Molting individuals

Molting individuals are characterized by the separation of molting cuticle at extremities: head and tail tip. The genital primordium structure of molting specimens is intermediate between structures of primordia described above for the juveniles of the J2—J4 stages.

Molt from J2 to J3 (n = 1). (fig. 5, 6).

L = 233 μm; a = 30; b' = 3.2; c = 16; c' = 3.6; GP = 8 μm; GP/GW = 2.6; GP/L = 3 %; (V) = 61 %.

In the center of primordium, 3—4 mitoses of somatic nuclei are distinct between germinal cells.

Molt from J3 to J4, male juvenile (n = 3) (fig. 3, *H*, *I*; 5).

L = 390 (386—395) μm; a = 25 (20—30); b' = 3.6—3.7; c = 13 (12—14); c' = 3.0 (2.2—3.8); GP = 18 μm; GP/GW = 3.4; Gp/L = 4.5—4.6 %.

The genital primordium is loop-shaped, with the anterior apex and both somatic and germinal parts reversed posteriorly; the somatic part is set off by constriction from germinal part and its tip is curved hook-like (fig. 3, *I*). The cloaca primordium is enlarged with a transparent inner cavity anterior to the rectum, containing transparent spicule outlines in 2 specimens. This molting phase is an indication of the orientation change of the somatic part, which in male juvenile J3 is located anteriorly and in J4 is shifted posteriorly, thus pulling a germinal part to the anterior direction (fig. 5, a late phase of J3—J4 molt).

Molt from J3 to J4, female juvenile (n = 2) (fig. 6).

L = 345—381 μm; a = 25—28; b' = 3.1—3.7; c = 11.5—13.3; c' = 3.8—3.9; GP = 17—21 μm; GP/GW = 2.8—3.5; Gp/L = 4.5—6.1 %; (V) = 61—65 %.

The somatic part of the genital primordium in its posterior part possesses more than 20 somatic nuclei, the germinal part in the anterior part of the primordium with 5—10 large germinal cells.

Molt from J4 to male (n = 1) (fig. 5).

L = 441 μm; a = 39; b' = 3.7; c = 15.2; c' = 3.6; GP = 224 μm; GP/GW = 25; GP/L = 52 %.

The somatic part of the genital primordium reaches the massive cloaca primordium, in which inner cavity transparent spicules outlines are distinct. The bursa is not formed, the tail is narrowly conical, with 9 μm long hyaline zone .

Molt from J4 to female (n = 1).

L = 524 μm; a = 38; b' = 4.7; c = 16.2; c' = 5.0; GP = 270 μm; GP/GW = 25; GP/L = 52 %; (V) = 73 %.

Vulva primordium is a massive cellular agglomeration with an inner transversal slit, but without opening outside. All sections of the genital system are distinct: the ovary of 30—40 germinal cells, the oviduct, empty spermatheca, crustaformeria, and the uterus with inner empty cavity.

Key to stages of individual development of ***Bursaphelenchus ulmophilus*** (fig. 5, 6)

1. Copulative structures completely developed, vulva or spicules present — Adults . . . . 2.

— Copulative structures absent — Juveniles . . . . 3.

2. Spicules present, tail tip with bursal flap, reversed, umbrella handle-shaped . . . . Male.

– Spicules absent, but vulva and developed female genital system present . . . . Female.

3. Genital primordium small, consists of two large germinal cells in center and two small apical nuclei at extremities (fig. 3, *B*), average body length less than 200 µm . . . . . Juvenile of the second stage (J2, sex not recognizable).

– Genital primordium consists of more than 10 cells, with 5 or more large germinal cells, average body length more than 300 µm — Senior juveniles . . . . 4.

4. Genital primordium length is 20 % or longer than body length — Juveniles of forth stage (J4) . . . . 5.

— Genital primordium length is 8 % or less than body length — Juveniles of third stage (J3) . . . . 6.

5. Cloaca primordium present around rectum; somatic zone of genital primordium not divided into sections (fig. 4, *A*) . . . . J4 Male.

— Cloaca primordium absent, somatic zone of genital primordium divided into sections, among them massive uterus primordium joined with vulva primordium at the ventral body wall (fig. 4, *B*) . . . . J4 Female.

6. Large germinal cells (5—8) in the posterior end of the genital primordium, cloaca primordium present around rectum (fig. 3, *F*, *G*) . . . . J3 Male.

– Large germinal cells (4—6) in the anterior part of the genital primordium, cloaca primordium absent (fig. 3, *C*, *D*, *E*) . . . . J3 Female.

Individual growth may be illustrated in diagrams (fig. 7) with three main parameters: the body length; the ratio between body length and the length of the pharynx till the posterior end of the pharyngeal gland lobe (b') and the ratio between the genital primordium length and the body length. The body grows faster than the pharynx in male and female juveniles (fig. 7, *B*). The maximum elongation of the body occurs between the third stage and the fourth stage (fig. 7, *A*). In the same period, the maximum elongation and differentiation of the genital primordium takes place in both male and female development lines (fig. 7, *C*). Juvenile body increases between molts and within developmental stages, which is evident in the size ranges of every stage (see table).

DISCUSSION

The embryonic development with hatching of J2 already molted in the egg may be considered as *ovoviviparity*. Because the sex of juvenile is distinct starting from the third stage (J3) it is considered that the common in Nematoda physiological sex determination takes place according to environment conditions for the second stage juvenile (J2). The fastest growth and genital primordium

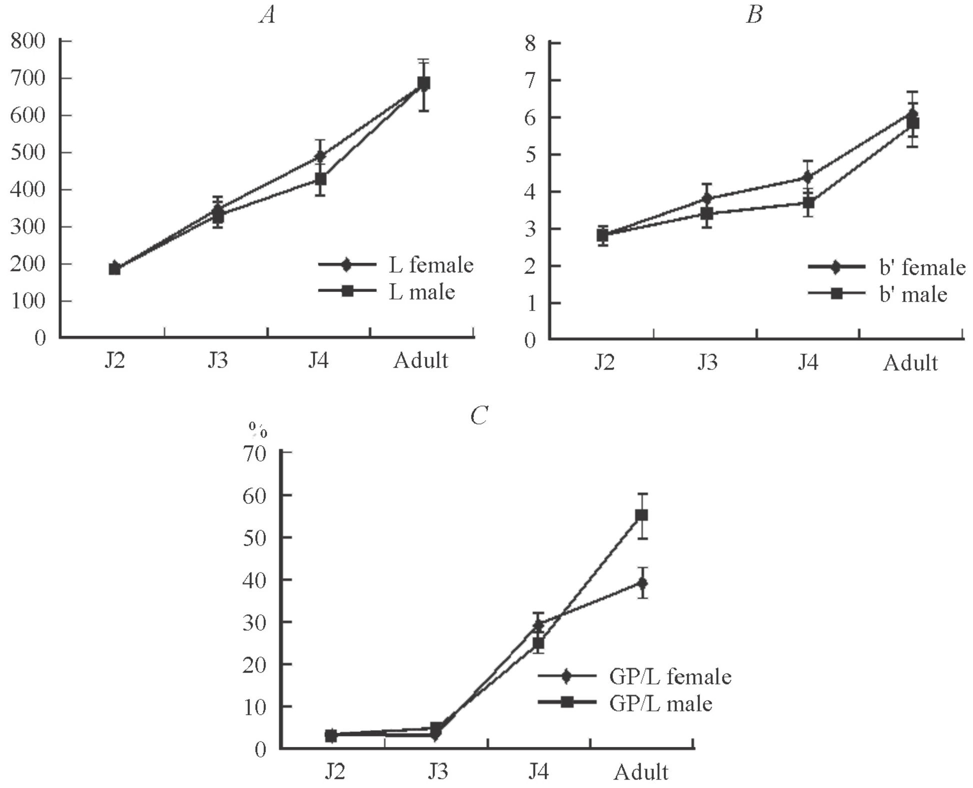

Fig. 7. Diagrams of the juvenile growth.
A — body length, *B* — ratio of body length to pharynx length (b'), *C* — ratio of genital primordium length to body length (GP/L, %). Standard deviation (STD) levels are marked for mean values.

differentiation takes place in the molt from J3 to J4 stages (fig. 7). Earlier study of the development of *Bursaphelenchus mucronatus kolymensis* (Ryss, Chernetskaya, 2009) gave the similar results: the first molt occurring in the egg shell and 4 stages outside of egg divided by molts: J2—J4 and adult worms. The only difference is the dauer juvenile stage. In *B. mucronatus* it is the fourth stage juvenile (Ryss, 2008). *Bursaphelenchus ulmophilus* dauers taken from the *Scolytus* spp. beetle under-elytra spaces are identical to the third stage juveniles of the propagative generation grown on *Botrytis cinerea* culture, according to the genital primordium structure (Ryss, et al, 2015 and the results of this study). Leaving the beetle surface, the *B. ulmophilus* dauers start to molt to the fourth stage juvenile with elongated and differentiated genital primordium, just in water (Ryss, et al., 2015). Differences between species are also manifested in the needle-like mucro on the tail tip, which is present in *B. mucronatus* in juveniles of J3 and J4 stages and in females (Ryss, Chernetskaya, 2009), but absent in adults and juveniles of *B. ulmophilus*.

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References

Fedorova S. M. 2010. [Epiphytoty of the Dutch elm disease in historical parks of Saint-Petersburg.]. In: Problemy ozeleneniya krupnykh gorodov. Conference Proceedings 9—11 February, 2010, Moscow. P. 72—77. (In Russian).

Hirschmann H. 1962. Life cycle of the *Ditylenchus triformis* (Nematoda: Tylenchida) with emphasis on post-embryonic development. Proceedings of the Helminthological Society Washington. 29 (1): 30—43.

Hirschmann H. 1971. Comparative morphology and anatomy. In: Plant parasitic nematodes. Vol. 1. B. M. Zuckerman, W. F. Mai, R. A. Rohde (ed). New York: Acad. Press. P. 9—63.

Hirschmann H., Triantaphyllou A. C. 1968. Mode of reproduction and development of the reproductive system of *Helicotylenchus dihystera*. Nematologica. 13 (4): 558—574.

Kalko G. V. 2008. [Dutch elm disease in Saint Petersburg.]. Mikologia i Fitopatologia. 6: 564—571. (In Russian).

Kalko G. V. 2009. [*Ophiostoma*-caused wilt of elms in Saint Petersburg.]. Zashchita i Karantin Rasteniy. 3: 48—49. (In Russian).

Ryss A. Y. 1981. [Morphogenesis of the female genital system in superfamily Tylenchoidea (Nematoda).]. Parazitologiya. 15: 533—542. (In Russian).

Ryss A. Y. 1988. [Root parasitic nematodes of the family Pratylenchidae (Tylenchida) of the world fauna]. L.: Nauka Publishers. 368 p. (In Russian).

Ryss A. Y. 2008. Entomophilic juveniles of the nematode *Bursaphelenchus mucronatus* (Nematoda, Aphelenchida, Parasitaphelenchidae) obtained from the cerambycid vector *Monochamus urussovi* (Coleoptera, Cerambycidae) under experimental conditions. Entomological Review. 88 (1): 108—114.

Ryss A. Y., Chernetskaya A. Y. 2009. [Life-cycle of *Bursaphelenchus mucronatus* Mamiya eт Enda, 1979 (Nematoda: Aphelenchida).]. Parazitologiya. 43(3): 206—224. (In Russian).

Ryss A. Yu. 2015. [The most simple techniques for detection and laboratory cultivation of woody plant wilt nematodes.]. Izvestia Sankt-Peterburgskoj Lesotehniceskoj Akademii. 211: 287—295. (In Russian).

Ryss A. Yu. 2017. The simplest «field» methods for extraction of nematodes from plants, wood, insects and soil, with additional description how to keep extracted nematodes alive for a long time. Parazitologiya. 15 (1): 57—67.

Ryss A. Yu., Polyanina K. S., Popovichev B. G., Subbotin S. A. 2015. Description of *Bursaphelenchus ulmophilus* sp. n. (Nematoda: Parasitaphelenchinae) associated with Dutch elm disease of *Ulmus glabra* Huds. in the Russian North West. Nematology. 17 (6): 685—703.

Polyanina K. S., Popovichev B. G., Ryss A. Yu. 2016. Elm wood nematodes as a threat to the urban woody plantations in the Russian North-West: the laboratory tests of woody plant host range and pathogenicity. The Kataev Memorial Readings — IX. Dendrobiotic Invertebrates and Fungi and their Role in Forest Ecosystems. Proceedings of the International Conference. Saint Petersburg (Russia), November, 23—25, 2016. D. L. Musolin and A. V. Selikhovkin (eds). Saint Petersburg. P. 90—93.

Ryss A. Y., Subbotin S. A. 2017. Coevolution of wood-inhabiting nematodes of the genus *Bursaphelenchus* Fuchs, 1937 with their insect vectors and plant hosts. Zhurnal Obshei Biologii. 78 (3): 13—42.